

Conservation Genetics of Rio Grande silvery minnow, *Hybognathus amarus*: genetic evaluation of wild and captive stocks, 1999 to 2003.

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Prepared by

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Executive Summary

Part I of this report examines use of ecological and genetic methods to evaluate effects of river fragmentation on the federally endangered Rio Grande silvery minnow. Once widespread and abundant throughout the Rio Grande, this species is now restricted to 5% of its historical range in a river reach fragmented by five dams. Adult census size (N) declined an order of magnitude from 1999 to 2001, and is currently $\sim 10^4$. Microsatellite and mtDNA analyses detected no spatial genetic structure, suggesting that Rio Grande silvery minnow is panmictic over its current range. Analysis of temporal genetic variation indicated substantial shifts in allelic frequencies from 1999 to 2001. Average genetic effective size (N_e) based on temporal-method estimation was about 70, and the ratio $N_e/N = 0.0012$ over the study period. Low values of N_e/N probably result from recent changes in demography related to extensive habitat fragmentation. Rio Grande silvery minnow produce passively drifting eggs and larvae subject to downstream transport by river currents, entrainment in diversion canals, and movement to unsuitable nursery habitats, which lead to low retention of yearly reproductive effort and poor recruitment. We propose that the interaction of passive dispersal in early life history stages and extensive habitat fragmentation sharply increases variance in reproductive success in remnant populations of Rio Grande silvery minnow, leading to low N_e compared to historical values. We conclude that river fragmentation has altered demographic and genetic dynamics of Rio Grande silvery minnow populations and reduced effective size to critically low values.

Part II extends results to genetic variation in wild Rio Grande silvery minnow sampled in 2002. A total of 389 fishes were fin-clipped and characterized for genetic

diversity at 10 microsatellite loci and one mtDNA locus. No appreciable genetic variation was found among river reaches, which is consistent with 1999-2001 data. Moreover, N_e estimated using temporal method comparisons with previous samples, remains critically low ($N_e=55$).

Part III considers the genetic effects of hatchery propagation in the Rio Grande silvery minnow. The purpose of this study was to determine whether captive propagation is maintaining the levels and patterns of genetic variation that are present in the wild population. To address this goal, variation in ten variable microsatellite loci and the mitochondrial ND4 gene was assessed in the wild population (2002) and in three hatchery stocks (2000, 2002 and 2003). The results indicate that the offspring of captively-spawned Rio Grande silvery minnow maintain similar levels of heterozygosity to the wild population but have reduced levels of allelic diversity. The converse is true of the captive population derived from wild caught eggs, which shows a marked reduction in heterozygosity whilst maintaining high allelic richness. Inbreeding values (F_{IS}) for the hatchery stocks show an increasing trend, with values twice that of the wild source population in the hatchery 2003 stock. These findings suggest that the goal of preserving the full extent of genetic variation present in the wild population in the hatchery stock is not being realized. The results indicate that the supplementation of the wild population with hatchery stock is likely to cause a further reduction in the effective population size. **Part IV** reports on egg collection activities for 2003, and **Part V** summarizes the major findings of this report and the implications for management and recovery of the Rio Grande silvery minnow.

Conservation Genetics of Rio Grande silvery minnow, Part I:

Conservation genetics of wild stocks 1999 - 2002

Introduction

Habitat destruction and fragmentation are arguably the most important general factors that drive global loss of species diversity (Vitousek *et al.* 1997, Young and Clark 2000). The direct demographic and genetic consequences of fragmentation for individual species and populations have been widely studied in terrestrial organisms through development of theory (e.g., Hanski 1999), manipulative experiments (e.g., Diffendorfer *et al.* 1995) and long-term demographic and genetic studies (e.g. Westemeier *et al.* 1998). Organisms restricted to lotic, freshwater habitats have received far less attention than terrestrial organisms despite the fact that most rivers in the developed world are severely fragmented by dams and other structures that impede free movement of organisms through river corridors (Behnke 1990; Dynesius and Nilsson 1994). Thus, there is a pressing need to understand the demographic and genetic consequences of river fragmentation for persistence of aquatic organisms (Dunham and Rieman 1999; Jager *et al.* 2001; Speirs and Gurney 2001).

At least two problems preclude direct application of knowledge derived from terrestrial ecosystems to riverine ecosystems. First, rivers are characterized by one-dimensional organization and unidirectional (downstream) movement of water (Speirs and Gurney 2001), whereas terrestrial systems usually have two-dimensional organization. As a consequence, fragmentation in rivers is easily accomplished with placement of a single dam sufficient for fragmentation (Jager *et al.* 2001). Second, migratory behaviors and life histories of riverine organisms often differ substantially

from well-studied terrestrial groups (e.g., birds and mammals), making it difficult to predict organismal responses to river fragmentation based on terrestrial models (Rieman and Dunham 2000).

In this study, we evaluated demographic and genetic effects of river fragmentation on the Rio Grande silvery minnow, *Hybognathus amarus*, a federally endangered fish species that is endemic to the Rio Grande. Before 1970, this species was abundant and widely distributed throughout the entire Rio Grande Basin. It is now restricted to a small (280 km) segment of the river that is fragmented by three water diversion dams and two major dams with reservoirs (Bestgen and Platania 1991). Diversion dams were designed primarily to divert water for agriculture uses, and have been implicated in the decline and/or extirpation of at least four fish species in the Upper Rio Grande, including the Rio Grande silvery minnow (Platania 1991). All four species produce semi-buoyant eggs that are passively transported by river currents (Platania and Altenbach 1998). There is evidence that young fish fail to recruit to breeding populations because eggs and larvae are entrained through water diversion structures and/or are transported downstream into unsuitable nursery habitats (e.g., Elephant Butte Reservoir) (Platania and Altenbach 1998; Luttrell *et al.* 1999). Even if larvae survive to adulthood, upstream return to natal sites is blocked by diversion dams.

Our investigation focused on spatial and temporal patterns of abundance and genetic diversity of remaining wild populations of Rio Grande silvery minnow in this fragmented landscape. The goals were twofold: (i) to provide baseline information about genetic diversity and abundance to help guide species recovery efforts; and (ii), to contribute empirical data toward a more general understanding of the interplay of

fragmentation, demography and life history, and genetic diversity for organisms that inhabit river ecosystems. We examined the interactions of demography, life history, and genetic diversity explicitly by calculating the ratio of the genetic effective population size (N_e) to adult census size (N). This ratio is expected to be unity in a Wright-Fisher idealized population (i.e., panmictic, 1:1 sex ratio, Poisson-distributed variance in reproductive success, stable population size – see Cabellero 1994; Wang and Cabellero 1999 for reviews). N_e/N is expected deviate from unity when idealized assumptions are violated, usually because of factors related to demography and life history. Using a variety of analytical approaches, we estimated N_e/N ratios for Rio Grande silvery minnow, and then interpreted these values in light of demographic and life history information gathered from intensive ecological monitoring of this endangered aquatic species.

Materials and Methods

Study species- The Rio Grande silvery minnow is a member of the Cyprinidae, the most species-rich family of freshwater fishes in the world (Helfman *et al.* 1997). The historical geographic range of this species comprised nearly the total length of the main stem Rio Grande and Pecos Rivers in New Mexico and Texas. Museum records and ichthyological accounts indicate that Rio Grande silvery minnow was among the most abundant fishes in this region. At present, the species is restricted to the middle portion of the Rio Grande in New Mexico and the species occupies only about 5% of its historical range. Dramatic reduction in geographic range size and abundance led to the listing of this fish as federally endangered in 1994 (U.S. Department of the Interior

1994).

Rio Grande silvery minnow are small fish (maximum total length 13 cm) that mature at age one, are short-lived (~90% individuals are estimated to die after first reproduction, Platania and Dudley, unpublished), and have effectively non-overlapping (i.e., discrete) generations. Despite small size, they are highly fecund with females producing up to 5,000 eggs per individual (Platania and Altenbach 1998). Fertilized eggs are about 1.0 mm in diameter when broadcast into the water column during spawning, but quickly swell with water to about 3.0 mm and become semi-buoyant. This reproductive strategy results in passive drift of eggs and larvae with river currents for about three to five days depending on river flow rate, water temperature, and developmental rate (Platania and Altenbach 1998). Drift distances are enhanced because adult silvery minnows spawn during elevated river flows in springtime, usually in late April or early May.

Study site-. This study was conducted in the middle Rio Grande, New Mexico, which is a 280-km river reach from Cochiti Dam to Elephant Butte Reservoir (Figure 1). The channel in the middle Rio Grande is shallow, sandy, and braided, and the river is constrained by levees that limit extent and duration of seasonal flooding associated with springtime snowmelt and precipitation. Average annual precipitation is low (<25 cm/year) and climatic conditions are semi-arid to arid throughout the basin. Three water diversion structures (from north to south Angostura Diversion Dam, Isleta Diversion Dam, and San Acacia Diversion Dam) divide the middle Rio Grande into four reaches: (i) the Cochiti reach, (ii) the Angostura reach, (iii) the Isleta reach, and (iv) the San Acacia reach (Figure 1). Rio Grande silvery minnow are extremely rare in the Cochiti reach, and

thus our study focuses on the latter three reaches.

Ecological Monitoring and Census Size Estimation- Rio Grande silvery minnow (and the associated fish community) were monitored bimonthly from February 1999 to December 2001 at 15 localities in the middle Rio Grande (Figure 1). Fishes were collected by rapidly drawing a two-person 3.1 m x 1.8 m small mesh (0.5 cm) seine through various mesohabitats, with about 15 to 20 seine hauls taken at each site. Catch-per-unit-effort (CPUE) was calculated as the number of fish collected per m² seined.

Adult census size (N) was determined by using sample station CPUE values from February and April of 1999, 2000, and 2001. Rio Grande silvery minnow spawn primarily during spring, and most individuals perish following their first spawn, so these two months represented the time of year when breeding adults comprised the vast majority (if not all) of the population. Two estimates of N were made. The first was based on total number of individuals collected at a sampling locality and the length of stream sampled. This method provided a conservative estimate of N by assuming all fishes at the sampling locality were collected. For example, if 100 Rio Grande silvery minnow were collected at a site that was 500 m long, it was calculated that 0.2 individuals occurred per linear river meter (RM [m]). Total census size was calculated for each sampling locality as $((\text{individuals}/\text{RM}) * D)$.

Second, CPUE data and available wetted area (AWA [m²]) of the river were used to estimate total numbers of individuals between sampling points. The midpoint between sampling localities was used to delineate each sub-reach. AWA for each sampling locality was calculated as distance between upstream and downstream midpoints (D [m]) multiplied by stream width (W [m]). Total N was calculated for each sampling locality as

total CPUE*AWA. This calculation assumes densities of fish in each sub-reach would be similar to habitats seined at each sampling locality. Differences in CPUE between years for both February and April collections were determined using two-way analysis of variance (ANOVA) without replication.

Characterization of genetic diversity-. From 1999 to 2002, adult Rio Grande silvery minnows were sampled at a total of seven localities in the middle Rio Grande (Table 1, Figure 1). Each year, fishes were collected prior to spawning (December through March) by seining, occasionally with the aid of a backpack electrofishing unit. Fishes in 1999 and 2000 year classes were frozen whole, returned to the laboratory, and stored at -80°C. A small portion of the caudal fin was removed for subsequent DNA isolation. For the 2001 year class, captured fishes were anesthetized in MS-222 (Tricaine Methanesulfonate 200mg/L river water) at the capture site and a small portion of caudal fin was removed from each individual and preserved in 95% EtOH.

Total nucleic acids were isolated from air-dried fin clip samples using standard proteinase-K digestion, followed by single phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol extractions (Hillis *et al.* 1996). Nucleic acids were precipitated by addition of 2.5 volumes ice cold EtOH and 0.1 volumes 2 M NaCl, dried by vacuum centrifugation, and resuspended in 50µl of sterile H₂O. DNA isolates were screened for genetic variation at seven variable microsatellite loci (*Lco1*, *Lco3* – *Lco8*; Turner *et al.* in press) and a 295 base pair fragment of the protein-encoding mitochondrial (mt) DNA subunit four gene (ND4).

Microsatellite loci were amplified using fluorescently-labeled primers and products were detected using an ABI 377 automated sequencer. Multiplex polymerase

chain reaction (PCR) was employed for the following combinations of microsatellite loci: (i) *Lco3*, *Lco4*, *Lco5*, *Lco8* (1 µl 10X PCR buffer, 2mM MgCl₂, 200mM dNTPs, 0.25µM each primer, 0.375U *Taq* DNA polymerase), and (ii) *Lco6*, *Lco7* (1 µl 10X PCR buffer, 2.5mM MgCl₂, 200 mM dNTPs, 0.4µM each primer, 0.375U *Taq*). Locus *Lco1* was amplified singly (1 µl 10X PCR buffer, 2.5mM MgCl₂, 200mM dNTPs, 0.5µM forward and reverse primers, 0.375U *Taq*), with 94°C denaturation for 2 min, 25 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 52°C (*Lco1*), 50°C (*Lco3*, *Lco4*, *Lco5*, *Lco8*), or 48°C (*Lco6*, *Lco7*), and 30 sec extension at 72°C. Upon completion of PCR, 10 µl formamide/dye solution (62.5% formamide, 25% bromophenol blue, 12.5% ROX-350 commercially available size standard) was added to amplification products and this mixture was denatured for 2 min at 94°C. Electropherograms were analyzed with GENESCAN Software designed for use with the ABI 377 automated sequencer.

MtDNA ND4 gene fragments were amplified in 10µl reactions that contained 1µl template DNA (approximately 50 to 100ng/µl), 1µl 10X reaction buffer, 2mM MgCl₂, 200mM dNTPs, 0.5µM forward (5'- GAC CGT CTG CAA AAC CTT AA - 3') and reverse primer (5'- GGG GAT GAG AGT GGC TTC AA - 3'), and 0.375U *Taq*. PCR conditions were 94°C initial denaturation for 2 min, and then 25 cycles of 94°C 30 sec, 50°C 30 sec, and 72°C 30 sec. Variation among individual fragments was visualized using single-strand conformational polymorphism (SSCP – Sunnucks *et al.* 2000). Representative haplotypes from each gel (~20%) were verified by direct sequencing using a ABI BigDye Terminator cycle sequencing kit and an ABI 377 automated sequencing apparatus. Nucleotide sequences were aligned with the SEQUENCHER package and adjusted by eye.

Genetic analysis-. Microsatellite allele and genotype frequencies, gene diversities, and other summary statistics were tabulated using GENEPOP version 3.1d (Raymond and Rousset 1995) and ARLEQUIN (Schneider *et al.* 2000). Genotypes at each microsatellite locus, locality, and year-class sample were tested for departure from Hardy-Weinberg equilibrium (HWE) expectations using a modified Fisher's exact test for multiple alleles as implemented in GENEPOP. Microsatellite loci were tested for linkage disequilibrium across all possible pairs of loci.

We examined population substructure in microsatellite and mtDNA sets separately, using hierarchical analyses of molecular variance (AMOVA). These analyses were aimed at partitioning standardized genetic variance into portions attributable to differences among river reaches (F_{CT}), among localities within reaches (F_{SC}), and among individuals within localities (F_{IS}). We observed fragment length changes that were inconsistent with a strict stepwise mutation process in most microsatellites assayed, and thus we assumed an infinite alleles mutation model (under this model new alleles are expected to be different from extant alleles in a population – Nei 1987) and computed Weir and Cockerham's (1984) F -statistics as implemented in ARLEQUIN. For mtDNA, standardized genetic variances are reported as phi-statistics (Φ), which are similar to F -statistics but differ in that pair-wise evolutionary distances (in this case, the number of nucleotide differences) between haplotypes are required to calculate sums of squares in AMOVA.

Rivers are one-dimensional habitats, and so an isolation-by-distance model of gene flow (Wright 1943) may best describe population substructure in the Rio Grande silvery minnow. We tested for genetic isolation by distance using the method of Rousset

(1997). Microsatellite and mtDNA data were evaluated separately using ordinary least-squares regression, where the dependent variable was either values of $F_{ST}/(1 - F_{ST})$, or $\Phi_{ST}/(1 - \Phi_{ST})$ [mtDNA] calculated for all possible pairs of localities (after Rousset 1997). The independent variable was linear river distance (km). Degrees of freedom were adjusted downward to seven (one minus the number of original localities sampled) rather than total number of pair-wise comparisons to account for inflated Type I error rates associated with non-independence (Hellberg 1994). If an isolation-by-distance model of gene flow holds, then a statistically significant positive slope is expected from regression of genetic divergence on distance.

Estimation of short-term (variance) N_e . Temporal changes of allele frequencies were characterized to provide an estimate of the genetic effective population size, N_e . For each microsatellite locus, we used the so-called temporal method (Nei and Tajima 1981; Waples 1989) to compute standardized variance in shifts of allele frequency (F) across year classes (pooled across sampling localities) using the formula

$$F = \frac{1}{K} \sum_{i=1}^K \frac{(x_i - y_i)^2}{(x_i + y_i)/2 - x_i y_i}, \quad (1)$$

where K is the number of alleles, and x_i and y_i are frequencies of allele i at generations 0 and t , respectively (Nei and Tajima 1981). Mean \bar{F} was computed by weighting locus-specific F values by the number of independent alleles at each microsatellite locus and averaging across loci (Waples 1989). With discrete generations and diploid loci, (variance) effective size can be expressed as

$$N_e = \frac{t}{2\bar{F} - \frac{1}{2S_0} - \frac{1}{2S_t}}, \quad (2)$$

where t is the number of generations separating each sample, S_0 and S_t are sample sizes at generations 0 and t , respectively (Waples 1989 – Sampling Plan I), and the terms $1/2S_0$ and $1/2S_t$ represent the variance expected from sampling the population at both years. For mtDNA, we modified equation 2 to reflect haploid inheritance (Turner *et al.* 1999). Finally, we computed N_e/N by dividing the estimate of N_e by the harmonic mean of midpoint values of N determined from CPUE data computed pairwise across 1999, 2000, and 2001 generations.

Estimation of long-term (inbreeding) N_e - We used an analytical method based on coalescent theory of gene genealogies to estimate the parameter Θ , as implemented in the computer program FLUCTUATE (Kuhner *et al.* 1998). For a neutrally-evolving, haploid locus in a panmictic population of stable size,

$$\Theta = 2N_e\mu, \quad (3)$$

where μ is the per-site mutation rate (Kingman 1982). FLUCTUATE allows relaxation of the stable population size assumption, and permits simultaneous estimation of Θ and a rate of population growth (or decline) scaled in terms of the number of mutations, g .

FLUCTUATE uses a Markov Chain Monte Carlo (MCMC) with importance sampling algorithm (Kuhner *et al.* 1995; Kuhner *et al.* 1998) to find the gene genealogy (i.e., the path of descent of a sample of genes from a single common ancestor), branch lengths, and parameters (Θ , g) that have the highest posterior joint probability under specific prior assumptions and a model of evolution (in this case the model of Felsenstein 1981). FLUCTUATE runs were set as follows: searches were initiated with a starting value of Θ based on Watterson's (1975) estimator; nucleotide frequencies were set to observed values; transition/transversion ratio was set to a value of two; and searches included ten

short chains with 10000 steps each and four long chains with 100000 steps each.

Separate runs were conducted where population size was assumed constant (i.e., g was not a parameter in the model), and where population size was free to change under a model of exponential growth (or decline). The approach employs likelihood functions to maximize probabilities and is computationally intensive, thus, our analysis focused only on the mtDNA ND4 fragment data, which was pooled across localities and year classes. There are at least two features of mtDNA that reduce the complexity of coalescent-based analysis; (i) mutations are quantified at the nucleotide sequence level and can be relatively easily modeled, and (ii) there is little or no recombination of the mtDNA.

To estimate long-term N_e from Θ , we assumed that the per-site mutation rate for the ND4 fragment is similar to other vertebrate mitochondrial genes and used the value $\mu = 2 \times 10^{-8}$ (Brown *et al.* 1979; Garrigan *et al.* 2002), which is equivalent to a substitution rate of 1% per million years. We substituted estimates of Θ from FLUCTUATE and μ into equation 3 to compute the long-term, inbreeding (female) effective population size, N_{eff} . If sex-ratio is unity, then long-term $N_e = 2 N_{eff}$.

Results

Ecological monitoring and estimation of N-. Adult census size (N) of Rio Grande silvery minnow was estimated to range between 26,715 and 1,389,419 during February 1999 based on stream length and available wetted area, respectively. Estimates in April 1999 were similar (range 25,103 to 1,066,328) to those in February 1999. Estimates of N ranged from 12,817 to 535,372 in February 2000 and from 15,181 - 617,158 in April

2000. Estimates of N ranged from 2,239 to 66,938 in February 2001 and from 1,704 to 54,485 in April 2001.

Mean CPUE was significantly different between years for February collections ($n = 15$; $F = 3.63$; $p < 0.05$) and nearly significantly different between years for April collections ($n = 15$; $F = 3.22$; $p = 0.055$). Pair-wise comparisons of 1999 and 2001, for both February and April collections were significantly different ($p < 0.05$). Mean CPUE declined from 6.52 ± 3.14 (SE) to 0.30 ± 0.11 (SE) between February 1999 to 2001 and from 4.80 ± 2.47 (SE) to 0.26 ± 0.13 (SE) between April 1999 to 2001.

Characterization of genetic diversity-. Amplification of seven microsatellite loci yielded clearly resolvable and consistent PCR products amenable to scoring with GENESCAN software. The number of distinct alleles identified at each locus ranged from eight to 46 when tabulated across all localities (Table 2). Statistical testing revealed significant departures from Hardy-Weinberg equilibrium, with an excess of homozygotes for all loci examined except *Lco5*. The weighted average inbreeding coefficient (F_{IS}) over all loci was 0.129. There was no evidence of linkage disequilibrium at nominal $\alpha = 0.05$ among loci when compared in pair-wise fashion across all loci. Expected and observed heterozygosity (calculated using the method of Nei, 1987) and mean number of alleles per locus are summarized by sampling locality in Table 2.

Nucleotide sequencing and SSCP characterization of the ND4 region of mtDNA yielded 13 distinct haplotypes from a total of 249 adult individuals sampled (Table 2). Haplotype diversity was lower than average gene diversity observed for microsatellites (Table 2). The most common haplotype (A) was present in 75% of individuals. Two haplotypes (D and F) were present at a frequency greater than 5%, and remaining

haplotypes occurred either at low frequency (< 5%) or were singletons. MtDNA – ND4 nucleotide sequences are deposited in Genbank under accession numbers XXXXXX—XXXXXX. Microsatellite and mtDNA data sets are available online at msbfishes.unm.edu.

Spatial genetic structure–. Hierarchical AMOVA indicated that a small but statistically significant proportion of genetic variation was attributable to differences among sampling localities within the San Acacia Reach (microsatellites $F_{SC} = 0.012$, $p < 0.001$; mtDNA $\Phi_{SC} = 0.027$, $p = 0.04$). No significant genetic variation was attributable to differences among Angostura, Isleta, and San Acacia reaches ($F_{CT} = 0.0004$, $p = 0.44$; $\Phi_{CT} = 0.037$, $p = 0.19$). There was no evidence for an isolation-by-distance model of gene flow based on regression analyses (Figure 2).

Temporal genetic structure and N_e/N -. Temporal shifts in allele/haplotype frequencies were observed across three generations of Rio Grande silvery minnow in the wild. For microsatellites, weighted mean standardized variance of allele frequency shifts (corrected for finite sample sizes by subtracting sampling variance terms as in the denominator of equation 2) was $\overline{F'} = 0.032$ when averaged across three values obtained in pair-wise comparisons between generations. This value is three times larger than variance attributable to differences among localities within reach (F_{SC}), and two orders of magnitude larger than differences among river reaches (F_{CT}). This value of $\overline{F'}$, when substituted into equation 2, corresponds to an average value of $N_e \approx 70$. Comparable temporal frequency shifts were observed in MtDNA-ND4 haplotypes, but estimates of N_e were slightly larger than those obtained for microsatellites for 1999 vs. 2000, and 2001 vs. 2002 comparisons (Table 3). One comparison, 1999 vs. 2001, yielded an estimate of

N_e that was indistinguishable from a population of infinite size (sensu Waples 1989).

Genetic effective size was three to four orders of magnitude smaller than harmonic mean adult census sizes in the middle Rio Grande, and consequently, the ratio N_e/N was much smaller than the expected value for an idealized population (Table 3).

Long-term (coalescent-based) estimates of N_e . Coalescent-based, genealogical analyses of mtDNA ND4 fragments indicated that $\Theta = 0.016$ under the assumption that Rio Grande silvery minnow exists in a stable (i.e., non-growing, $R_0 = 1$) population. Approximate lower- and upper-bound 95% CIs about Θ were 0.014 and 0.020, respectively. When exponential growth (or decline) was incorporated into the model, Θ was equal to 0.020 (lower- and upper bound 95% CIs were 0.019 and 0.020, respectively), and growth parameter (g) was equal to 854.5 (lower- and upper-bound 95% CIs were 555.7 and 1153.2, respectively). Recall that g is scaled by the mutation rate (Kuhner *et al.* 1998), and so when $\mu = 2 \times 10^{-8}$, the corresponding reproductive rate (R_0) is approximately 1.0017. Substituting Θ and μ into the equation 3, solving for N_{ef} , and then multiplying by two yielded: (i) long-term N_e estimated under an assumption of population stability equal to 791,367 (lower- and upper-bound 95% CIs were 676,248 and 1,014,795, respectively), and long-term N_e estimated under the exponential growth model was equal to 1,024,650 (lower- and upper-bound 95% CIs were 929,950 and 1,210,262, respectively).

Discussion

The task of identifying specific factors that impact genetic diversity of imperiled populations is often complicated by incomplete understanding of ecological parameters

such as population numbers, age structure, migration dynamics, and, most importantly, a lack of baseline genetic data that precedes major disturbance and fragmentation events. Our investigation of the endangered Rio Grande silvery minnow addressed these problems by: (i) documenting spatial and temporal patterns of genetic diversity over three generations in its present geographic range; (ii) evaluating the relationship of spatial and temporal genetic changes to changes in adult census numbers over three generations, and (iii) estimating effective size using computational methods that reflect contemporary and historical population dynamics, respectively. In the case of Rio Grande silvery minnow, the combination of ecological and temporal genetic information is essential to infer the nature and magnitude of processes that shaped genetic diversity in the past, and at present, in this species.

Ecological monitoring and estimation of N . Ecological monitoring of Rio Grande silvery minnow indicated temporal fluctuations in adult census size, most notably, an order of magnitude decrease (from 10^5 to 10^4) in 2001. Adult numbers probably decreased in response to prolonged drought and flow intermittency that began in summer 2000 and has continued through 2003. Estimates of N based on conservative analysis of catch per unit effort data, indicated that it is highly unlikely that adult census numbers were less than 10^3 , and probably have not been lower than 10^4 over the study period.

Spatial patterns of genetic diversity. Extensive river fragmentation in the middle Rio Grande does not result in appreciable spatial genetic structure among river reaches, probably for two reasons. First, there is enormous potential for unidirectional (i.e., downstream) gene flow at all life stages including in drifting egg and larval stages. If this scenario was the exclusive cause of spatial genetic patterning in the Rio Grande, then

upstream reaches should exhibit low genetic diversity relative to downstream reaches because dams preclude upstream migration, and an isolation-by-distance model of gene flow would be expected. Neither observation was realized in our data set (see Table 2), most likely because of recently implemented management strategies for the Rio Grande silvery minnow. Beginning in 1996, a severe drought year, the US Fish and Wildlife Service began rescuing and transporting wild adult fishes from drying areas in the San Acacia river reach to wetter Angostura and Isleta reaches (Figure 3). In 2000, a plan for propagating and/or rearing silvery minnows in captivity for subsequent release into the wild (beginning in 2001) was instituted. Both efforts continue to the present. Adult brood stock and/or wild caught eggs for hatchery propagation are derived from localities below San Acacia diversion dam. An excess of 500,000 hatchery-produced and a much smaller number of wild fishes have been repatriated into the wild north of San Acacia in the Angostura and Isleta reaches (J. Brooks – US Fish and Wildlife Service, pers. comm.). This management strategy provides ample opportunity for gene flow from lower reaches to upper reaches, and movement of repatriated genes back to downstream reaches through movement of eggs, larvae, and adults over diversion dams. A conceptual model for gene flow is presented in Figure 3, where the effects of strict downstream movement of genetic material in the wild is counteracted by movement of wild and hatchery-reared fishes to upper river reaches.

Temporal Genetic Diversity and short-term N_e . Population genetic theory suggests that critical losses of genetic diversity and concomitant accumulations of deleterious alleles are not expected over ecological time scales unless breeding adult census numbers are 10^2 or lower (Luikart *et al.* 1998; Lynch and Gabriel 1990), assuming

the genetic effective population size (N_e) is a function of the adult census size (N). In an idealized population these numbers are equal, and the ratio N_e/N is one. Empirical and theoretical examination of the ratio N_e/N for real organisms suggests that this number is rarely < 0.1 and usually hovers around $0.25 - 0.5$ under a variety of life histories, mating systems, and demographic scenarios (Nunney and Elam 1994; Frankham 1995a). Rio Grande silvery minnow populations are expected to harbor substantial genetic diversity by virtue of relatively large N .

Observed N_e for Rio Grande silvery minnow, estimated using the temporal method, was order 10^2 and three to four orders of magnitude lower than harmonic mean N depending on the sampling interval (1999-2001). Moreover, inbreeding coefficients (F_{IS}) were positive for six of seven microsatellite loci examined, which is consistent with the low N_e . Observed $N_e/N \approx 0.0012$ (averaged across generations using Kalinkowski and Waples 2002 – equation 2) is much lower than expected for an idealized population, and lower than empirical and theoretical estimates of $N_e/N \approx 0.1 - 0.5$. Low observed N_e/N suggests that Rio Grande silvery minnow populations will experience critical losses of allelic diversity and heterozygosity in subsequent generations, despite relatively large adult census size.

Of the possible factors that can lower N_e/N below idealized expectations (Cabellero 1994; Wang and Cabellero 1999; Turner *et al.* 2002; Waples 2002), two appear plausible for explaining low N_e/N in Rio Grande silvery minnow. First, ecological monitoring data indicates that adult census number has decreased substantially over the past three generations. The temporal method for estimation of N_e is sensitive to such fluctuations in population size when they occur over the sampling period (Luikart *et al.*

1998). However, if we calculate harmonic mean midpoint census sizes of 10^4 and 10^5 for two generations and solve for the number necessary to account for $N_e \approx 70$, then an adult census size of 24 individuals is required in the third generation. Ecological monitoring has never yielded so few fish in the wild, and thus, it seems unlikely that fluctuating population size alone can account for low N_e observed in Rio Grande silvery minnow.

We hypothesize that a second factor related to extremely large variance in reproductive success among individuals is driving N_e to low values in Rio Grande silvery minnow. Large variance in reproductive success has been posited to explain low N_e/N in species with mortality schedules best characterized by a Type III survivorship curve (Hedgecock 1994; Turner *et al.* 2002). Type III survivorship is characteristic of species with enormous capacity for reproduction, low allocation of energy per individual offspring, and extremely high mortality in early life stages. Species with this suite of life-history traits, like the Rio Grande silvery minnow, may be subject to a "sweepstakes" process whereby eggs and larvae experience high mortality as they disperse into highly heterogeneous environments (Hedgecock 1994). It has been proposed that such recruitment dynamics could produce large variances in reproductive success far in excess of Poisson expectation, ultimately yielding very low N_e/N (Hedgecock 1994). Such a mechanism is likely to be affecting N_e/N in the contemporary Rio Grande silvery minnow population. Production of offspring is lost each season as eggs and larvae are swept from natal spawning sites by river currents, entrained in diversion dams, and transported to unsuitable nursery and rearing habitats. Empirical studies in the middle Rio Grande of the dynamics of drifting particles that mimic eggs in size and density suggest that a very small fraction of eggs are retained in any river reach and that the vast majority of

production is swept into Elephant Butte Reservoir (RK Dudley – personal observation). We propose that extensive mortality of offspring and resulting high variance in reproductive success among individuals is a direct result of the interaction of the pelagic early life history of Rio Grande silvery minnow and fragmentation of its remaining habitat. As a consequence of this interaction, very few breeding pairs leave the majority of offspring that reproduce in the subsequent generation.

Comparisons of temporal method estimates of N_e between microsatellites and mtDNA suggest that another factor related to variance in reproductive success, skewed sex-ratio, may also play a small role in lowering N_e/N . On average, effective size generated for mtDNA was larger than for nuclear-encoded microsatellites, which could possibly indicate smaller variance of reproductive success for females. There are two important caveats to this interpretation. First, determining the sex of wild caught fishes is difficult because they are not obviously sexually dimorphic until females are fully ripe, and so there is no direct information on sex ratio in the wild. Second, statistical power to estimate N_e and 95% confidence intervals via the temporal method is sharply curtailed in the mtDNA dataset because sample sizes are smaller, and there are fewer independent alleles available to estimate standardized variances of frequency shifts (Waples 1989; Turner *et al.* 2001).

Comparison of short and long-term N_e . Temporal and coalescent-based methods provide different estimates of genetic effective size, N_e . The temporal method estimates variance effective size based on changes in allele frequencies over the time interval in generations between sampling periods (Waples 1989), and thus provides an estimate of N_e for a contemporaneous population. Conversely, the coalescent-based method

estimates inbreeding effective size (Orive 1993) and provides a long-term estimate of N_e integrated over the time to common ancestry of all alleles in the population (Avice 2000; Garrigan *et al.* 2002). Variance and inbreeding effective sizes are equal for a large population of constant size (Whitlock and Barton 1997), but differ substantially under other conditions such as fluctuating effective population size (Crow and Denniston 1988, Vucetich *et al.* 1997). Temporal-method estimates of N_e are very sensitive to population size fluctuations over the sampling interval and can be used to detect recent population bottlenecks (Luikart *et al.* 1998). Long-term estimates of N_e are not as sensitive to recent changes, but respond to historical changes in population size (Avice 2000). In our study, short- and long-term measures of N_e were used in concert to understand how recent river fragmentation has affected N_e in contemporary populations relative to historical populations of Rio Grande silvery minnow.

If life history and river fragmentation interact to drive N_e/N to low values in contemporary Rio Grande silvery minnow populations, then we would predict that effective size prior to fragmentation was much larger. Coalescent-based estimates of effective size estimates are on the order of 10^6 . It is not possible to calculate historical N_e/N directly because we are unsure of pre-fragmentation adult census size. However, if 10^5 fishes currently occupy 5% of the historical range, then we can estimate the historical census size to be on the order of 10 million fishes (20 times 5×10^5). This would put the historical N_e/N ratio of Rio Grande silvery minnow in the neighborhood of empirically derived estimates of $N_e/N \approx 0.1$ (Frankham 1995a). Values of $N_e/N \sim 0.001$ observed in present-day Rio Grande silvery minnow suggest that contemporary population dynamics are very different from those that impacted historical populations. Observed differences

of effective size between temporal method and coalescent-based estimators is consistent with the interpretation that recent river fragmentation has resulted in demographic changes that lower N_e to critical levels in contemporary Rio Grande silvery minnow populations.

Conservation biologists sometimes distinguish between demographic and genetic processes and their effects on the likelihood of persistence of an endangered species. In an influential paper, Lande (1988) argued that the time scale over which stochastic demographic processes can lead to extinction are generally much shorter than time scales required for extinction by genetic causes (e.g., accumulation of deleterious mutations, loss of fitness due to inbreeding), and thus, should receive highest priority for management. An important, but sometimes overlooked, point raised in Lande's paper was the emphasis on interactions of demographic and genetic factors in extinction. We have shown that genetic effective size in the Rio Grande silvery minnow is much smaller than expected based on adult census size, in fact, sufficiently small to warrant concern about extinction from genetic factors (Higgins and Lynch 2001). Our analysis further suggests that the underlying ecological factors that lower effective population size occurred relatively recently, and are related to demographic effects caused by an interaction of life history and extensive river fragmentation. Further refinement of our understanding of demography, life history, and genetic diversity will ultimately lead to deeper insight into the processes of biological extinction and conservation (Westemeier *et al.* 1998; Soulé and Mills 1998, Young and Clark 2000).

Conservation Genetics of Rio Grande silvery minnow, Part II:

Summary of genetic findings in 2002 wild stocks

Introduction

We have recently completed genetic screening of 389 Rio Grande silvery minnow, collected from Angostura, Isleta, and San Acacia Reaches from December 2001 to March 2002. Fishes collected in 2001 were adult fishes that were considered to be potential spawners in 2002. We assayed seven microsatellites developed in our laboratory and used in **Part I** (see Appendix I), and three microsatellites developed for the eastern stoneroller, *Campostoma anomalum* (*Ca1*, *Ca6*, *Ca8* – Dimsoski *et al.* 2000). MtDNA ND4 haplotypes were determined for 377 individuals using SSCP. Nucleotide sequencing of selected individuals was conducted to ensure the accuracy of haplotype designations. We report results based on 10 DNA microsatellite loci and variation in 295 base pairs of ND4.

Materials and Methods

See materials and methods for **Part I** (pages 10 – 18).

Results and Discussion

Several important results, consistent with findings from 1999 to 2001, emerge from this effort. Firstly, inbreeding coefficients ($F_{IS} = 0.223$) are higher, on average, than previous sampling periods (Table 4). Positive inbreeding coefficients indicated a deficiency of heterozygotes, which is expected when genetic effective size is small.

A total of eight ND4 haplotypes were identified. The most prevalent haplotype

(A) was present in over 50% of individuals. Haplotypes C and D were also common. The remaining haplotypes occurred at a low frequency (<5%) (Table 5).

We found no evidence of significant spatial genetic structure attributable to differences among river reaches (based on microsatellite data). Standardized genetic variance attributable to differences among reaches, $F_{CT} = 0.013$, was remarkably consistent with findings in **Part I**. Our results suggest that Rio Grande silvery minnow is panmictic over its current geographic range in the Middle Rio Grande. The management implications of this finding are discussed in **Part V**.

Genetic effective size N_e , estimated via the temporal method (eq. 1-2, **Part I**), remained small in wild Rio Grande silvery minnow populations. Results of pairwise estimates of N_e among generations from 1999 to 2002 are presented in Table 6. $N_e = 105$ when averaged across all possible comparisons of 2002 fish with previous generations. High inbreeding coefficients, and low N_e suggest that Rio Grande silvery minnow will experience critical losses of genetic diversity over the next few generations, unless steps can be taken reduce or eliminate habitat degradation and fragmentation.

**Conservation Genetics of Rio Grande silvery minnow, Part III:
Genetic Effects of Hatchery Propagation (2000- 2003)**

Introduction

The precipitous decline of the wild population and the lack of recruitment of Rio Grande silvery minnow in the summer of 2000 prompted the collection and placement of eggs and wild fish in propagation facilities in 2000 (Davenport and Brooks 2003). Captive-rearing can improve the survival of early life stages by reducing mortality imposed by factors including predation, food availability, and catastrophic events. The primary goal of supportive breeding is to augment the wild population with hatchery-raised fish to increase the census population size and thus prevent the extinction of the species in the wild. Hatchery stocks also provide refugial populations and guard against the species extinction in event of a catastrophic loss of the wild population. Propagation should also provide fish that contribute to the long-term viability of the wild population and so should strive to maintain the species genetic diversity. The loss of genetic variation can reduce a species ability to respond to environmental changes. Between 2000 and 2003 over 500,000 hatchery-reared and propagated Rio Grande silvery minnow were released in the middle Rio Grande (Remshardt 2002, Davenport and Brooks 2003). Although the census size of the wild population may be increased by supportive breeding and subsequent introductions, there are risks associated with such measures. Detrimental genetic impacts include introduction of non-adaptive traits (Lynch *et al.* 1995), reduction in the effective population size (Ryman and Laake 1991), inbreeding depression (Frankham 1995b) and maladaptive behavioral changes. Hatchery fish may be

depauperate of overall genetic variability, and this deficiency may reduce the variability in the wild recipient population (Tringali and Bert 1998). This will be most evident if few founders are used to as brood-stock. For a hatchery population to retain 99% of the heterozygosity of the wild population 50-200 effective founding breeders has been suggested (Ryman and Stahl 1980, Frankel and Soulé 1981). The loss of heterozygosity can lead to lower viability and fecundity (inbreeding depression) (Falconer 1981, Ralls and Ballou 1983). Inbreeding depression occurs when there is a population size reduction where the frequency of deleterious recessive traits can change. Some of these alleles will be lost but some may drift to high frequencies in the population, be expressed as homozygotes thereby reducing individual fitness.

The current trend, particularly in fish, places the hope of endangered species recovery in captive propagation and supportive breeding (Hedrick *et al.* 2000a). It is imperative therefore, that the genetic effects (in the hatchery fish and in the wild recipient population) of such measures be considered. To date, the majority of studies into the effects of hatchery propagation have focused on species that are important to the fisheries industry, particularly salmonids (Hindar *et al.* 1991; Wang *et al.* 2002). These species have very different life histories to those of many threatened non-salmonid fish species. Of the 114 threatened and endangered fishes in North America, over a third are members of the Family Cyprinidae (minnows) and at least five are being captively-propagated in recovery efforts (US FWS 2003). The aim of the present study is to evaluate the genetic effects of hatchery propagation in Rio Grande silvery minnow.

Materials and Methods

Sampling- Fin clips were taken from 389 Rio Grande silvery minnow collected from three reaches (Angostura, Isleta and San Acacia) of the Rio Grande in December 2001 as outlined in Part 1.

Rio Grande silvery minnow eggs were collected from the San Acacia reach during the peak spawning period which usually occurs over a week in mid to late May in response to increased flows from spring runoff (Platania and Dudley 1999, 2000). Eggs are reared in propagation facilities and released in the Angostura reach of the Rio Grande prior to spawning (between December and March) (Davenport and Brooks 2003). Fish are tagged using visible implant elastomer prior to release, enabling them to be monitored and distinguished from wild fish (Remshardt 2002). Three years of hatchery propagated *Rio Grande silvery minnow* were considered. Year 2000 fish (referred to hereafter as Hatch 2000) were raised from captively-spawned wild caught adults (collected from the San Acacia reach). Year 2002 (Hatch 2002) and 2003 (Hatch 2003) were raised from wild caught eggs (from the San Acacia reach). Fin clips were taken from adults prior to their release. These were stored in 95% ethanol. DNA was extracted from fin clips using standard protocols.

Individuals were screened for genetic variation at ten microsatellite loci (Lco1, Lco3, Lco4, Lco5, Lco6, Lco7, Lco8 (Turner *et al.* In Press; Appendix I), CA1, CA6, CA8 (Dimsoski *et al.* 2000). Microsatellite loci were amplified using fluorescently labeled primers. The following microsatellites were amplified (in a 10 μ L reaction volume) using multiplex PCR: Lco3, Lco4, Lco5 (1X PCR buffer, 2mM MgCl₂, 200mM dNTPs, 0.40 μ M each primer, 0.375 units TAQ polymerase), Lco6, Lco7 (1X PCR buffer,

2.5mM MgCl₂, 200mM dNTPs, 0.40μM each primer, 0.375 units TAQ polymerase), CA1, CA6 (1X PCR buffer, 2mM MgCl₂, 200mM dNTPs, 0.40μM each primer, 0.375 units TAQ polymerase). The remaining microsatellites were amplified alone (Lco1, Lco8, CA8) (1X PCR buffer, 2.5mM MgCl₂, 200mM dNTPs, 0.50μM each primer, 0.375 units *Taq* polymerase). PCR cycling condition were: one denaturation cycle of 94°C for 2 mins followed by 30 cycles of 94 °C for 20 sec, 48°C (Lco6, Lco7, CA1, CA6) or 50°C (Lco3, Lco4, Lco5, Lco8) or 52°C (Lco1, CA8) for 20 sec, 72°C for 30 sec. Prior to electrophoresis 1.2μL of PCR product was mixed with 1.2μL of a solution containing 62.5% formamide, 25% bromophenol blue, 12.5% ROX350 size standard and denatured at 94°C for 2 minutes and placed on ice. Products were electrophoresed on an ABI377 automated sequencer and analyzed with Genescan software.

A 295 base pair fragment of the mitochondrial ND4 gene was amplified as outlined in Part 1.

Data Analysis-. Rio Grande silvery minnow releases pelagic eggs (Platania and Altenbach 1998). It is predicted that drifting eggs collected from the lower reach (San Acacia) will represent the genetic diversity seen over the entire population. This prediction is based of the empirical study of the transport of artificial eggs that mimic the size and density of Rio Grande silvery minnow eggs (Dudley and Platania 2000). Samples from the three reaches of the middle Rio Grande were pooled for comparisons with the hatchery fish.

Microsatellite data were analysed using GENETOP Version 3.1d (Raymond and Rousset 1995) and FSTAT Version 2.9.3.2. For each population and locus the gene diversity, number of alleles, allelic richness (based on the minimum sample size of 54)

and F_{IS} were calculated (FSTAT). Nei's estimation of heterozygosity was obtained for each locus and over all loci. The following analyses were conducted using GENEPOP. Each locus and population was tested for deviations from Hardy-Weinberg expectations (Rousset and Raymond 1995). Global tests for linkage disequilibrium were performed for all pairs of loci (Markov chain parameters were dememorization 5000, batches 500 and iterations per batch 5000). Population differentiation (genic) was tested using Fisher exact test (Raymond and Rousset 1995) between all populations and for each loci where the null hypothesis tested is that the allelic distribution is identical across populations. Genotypic differentiation amongst populations was tested where the null hypothesis is that the genotypic distribution is identical across populations. Markov chain parameters were dememorization 1000, batches 100 and iterations per batch 1000. An unbiased estimate of the P-value of a log-likelihood (G) based exact test was used (Goudet *et al.* 1996). ND4 data was analyzed using Arlequin software (Schneider *et al.* 2000). Genetic distances were obtained using the Kimura-two parameter method.

Results

Microsatellites -- Genetic diversity- The 10 microsatellite loci considered were moderately to highly polymorphic with the number of alleles ranging from a minimum of 8 at Lco5 to 61 at Lco1 (Appendix II). Allelic richness was the greatest for Lco 1, Lco5 and CA8 in the wild population (Appendix II). With the exception of Lco8 allelic richness was the poorest for the Hatch 2000 population. When the three river reaches are compared for the wild population there is a decline in allelic richness for four loci (Lco1, Lco5, Lco7, Lco8) from San Acacia to Angostura. This trend is reversed for Lco6 and

CA1. Significant linkage disequilibrium was identified between five pairs of loci (from a total of 44 comparisons): Lco3 with Lco4 ($P < 0.05$), Lco5 with Lco3 and Lco4 ($P < 0.05$), Lco6 with Lco7 and Lco8 ($P < 0.05$). A significant deficiency of heterozygotes was observed in all populations and for nearly all loci. In the Hatchery 2000 population allele frequencies at Lco3, Lco5, Lco6 and Ca6 did not differ significantly from Hardy-Weinberg expectations. Over all loci F_{IS} values ranged from 0.199 in the Hatch 2000 population to a high of 0.415 in Hatch 2003 population (Figure 4, Table 7). F_{IS} for the Hatch 2003 stock was twice that seen in the wild population (0.223). Comparisons of allele and genotypes frequencies between the wild population and the hatchery 2003 population revealed significant differences for all loci with the exception of CA1 and CA8 (and CA6 for genotypic frequencies).

Mitochondrial DNA- ND4 Genetic Diversity- Eleven ND4 haplotypes were detected among 670 individuals. These haplotypes differed by between one and nine transitions with sequence divergence ranging from 0.34% to 2.43%. In all populations haplotype A was the most common (Table 8). Four rare haplotypes were identified (J, N, O, P) which were present in single individuals.

Discussion

Captive propagation should aim to maintain the species genetic diversity to ensure the long-term viability of the wild population. The conservation of genetic diversity in a population requires that the composition (allelic diversity and heterozygosity), distribution (spatial distribution and heterogeneity) and the function of the variation is maintained (Brown *et al.* 2000). Several findings presented here suggest that the goal of

retaining genetic variability in the captively-propagated Rio Grande silvery minnow is not being realized.

Results indicated that the captively-spawned fish maintain heterozygosity but are depauperate of allelic richness whilst fish raised from wild caught eggs have lost heterozygosity whilst maintaining allelic diversity. The loss of alleles (microsatellite and ND4 loci) from captively-spawned Rio Grande silvery minnow is not surprising as most rare alleles will not be sampled when the brood-stock is founded by relatively few individuals. The probability of retaining alleles is proportional to their frequency in the population so rare alleles are least likely to be retained during population bottleneck events. If rare alleles are sampled they are likely to be lost rapidly by genetic drift (Lacy 1987) as the probability of their retention is directly proportional to the effective population size (Allendorf 1986).

Observed heterozygosity of the captively-spawned population is equivalent to that seen in the wild population. This is also consistent with the observation that rare alleles are lost more rapidly than heterozygosity during bottleneck events (Lacy 1987). The relatively low inbreeding co-efficient observed in the captively-spawned stock may be a result of reduced variance in reproductive success compared to the wild population.

An advantage of using wild-caught eggs as hatchery stock is that they should be a more representative sample of the alleles present in the wild population because of the large number of eggs that can be collected. At several loci the allelic richness in the hatchery (2002, 2003) stocks actually exceeded that observed in the wild population. This observation may be explained by the increased survival of early life stages of captively-reared Rio Grande silvery minnow when compared to those in the wild. It also

points to the probability of relaxed selection in the captive environment. This has the potential to lead to the proliferation of deleterious alleles in the captive fish (Lynch and O'Hely 2001).

There is an obvious trend of declining heterozygosity in the hatchery stocks (2002, 2003) with the 2003 stock retaining only about 78% of the heterozygosity of the parental source population. The loss of heterozygosity and allelic diversity in captive or refugial populations has been reported in a large number of fish species including the mosquito fish (*Gambusia affinis*) (Stockwell *et al.* 1996), cutthroat trout (*Onchorhynchus clarki*) (Allendorf and Phelps 1980) and Atlantic salmon (*Salmo salar*) (Cross and King 1983). The life history of the species concerned is a critical determinant of how the wild population will respond to the introduction of captively-raised fish. Regardless of the species, there is little doubt that supplementation has significant impacts upon the recipient population and that these impacts are often unpredictable (Hindar *et al.* 1991).

Rio Grande silvery minnow is a short-lived fish with few individuals surviving beyond 13 months (Propst 1999). The impact of supplementation is likely to be more extreme and rapid in Rio Grande silvery minnow than in long-lived species, with the hatchery-reared fish contributing immediately to the reproductive output of the wild population. If captively-reared fish (and their genetic variation) are over-represented in the wild population the effective population size may be reduced. This occurs as there is an inflated variance of family size between wild and hatchery stocks resulting from the increased survival of hatchery young (Wang *et al.* 2002). Additionally, the reproductive contribution of individual brood-stock may not be equal due to factors including incomplete mixing of sperm and eggs, sperm competition, variation in female fecundity,

differential survival of matings (Brown *et al.* 2000). Therefore the effective number of breeders in the captive population may be lower than predicted. High variances in reproductive success can accelerate the rates of genetic drift thereby reducing the effective population size. In Rio Grande silvery minnow there is evidence that the effective population size of the wild population is already very small (Part 1 and 2), thus, although a large number of eggs can be collected from the wild population, they may only represent the progeny of a small number of adults.

The reduced heterozygosity seen in the hatchery raised Rio Grande silvery minnow suggests that the assumption that eggs collected in the lower reaches represent the full extent of the genetic diversity in the wild population, may not be valid. Possible explanations include retention of eggs in the upper reaches by natural process or entrainment by diversion structures (Smith 1998). Although there is evidence that eggs can be transported from the upper to the lower reaches of the Rio Grande (Dudley pers. comm.), there will be mortality associated with downstream movement through diversion structures.

The trend of decreasing heterozygosity in the captively-propagated Rio Grande silvery minnow is a concern given that from the 2003 captive stock alone, 130 000 fish have been repatriated to the Rio Grande. If these fish spawn successfully it leads to the prediction that increasingly high F_{IS} values will be apparent in their offspring. The problems associated with the small effective size of the wild population may be compounded by the introduction of the hatchery-reared fish.

The detection of very few, closely related mitochondrial ND4 haplotypes in the 670 Rio Grande silvery minnow screened is consistent with a population that has

experienced bottleneck events. During bottleneck events the probability of retaining an allele is directly proportional to its frequency in the population (Allendorf 1986), hence rare alleles are more likely to be lost during such events. If severe population reductions occur in the northern reaches of the Rio Grande they can only be re-colonized by artificial translocations of individuals as diversion structures prevent upstream movement.

Although the introduction of hatchery-raised fish may temporarily increase the census population size, the status of the wild population of Rio Grande silvery minnow is unlikely to be improved unless the primary reasons for the populations continued decline are addressed.

Conservation Genetics of Rio Grande silvery minnow, Part IV:

Temporal genetic monitoring of pelagic eggs

Introduction

The Rio Grande silvery minnow is a pelagic spawner with semi-buoyant eggs (Platania and Altenbach 1998), which is a rare reproductive strategy among freshwater fishes. Consequently, much of the annual production of offspring is moved downstream with river currents after spawning, and implies that juveniles and adults historically migrated upstream, perhaps to natal sites in upper river reaches. The Rio Grande is now highly fragmented by dams and other diversion structures that impede upstream movement of fish.

We have shown in a long-term study of genetic variation in silvery minnow that genetic effective size N_e is three orders of magnitude smaller than census size in this species. The cause of low N_e/N appears to be associated with high variance in reproductive success among individuals, exacerbated by annual loss of reproductive output to entrainment over diversion dams and transport to unsuitable habitats. Loss of production would exert maximal effect on variance in reproductive success if genetic diversity of eggs is structured spatio-temporally (Hedgecock 1994), such that groups of genetically related eggs are subject to mortality that varies in space and time. We are testing whether genetic diversity of eggs collected at a single location from natural spawning varied among temporally-spaced samples. Preliminary genetic screening of eggs using mtDNA gene markers, SSCP, and nucleotide sequencing suggests strong temporal variability among egg samples. This finding implies high variance in

reproductive success is an important factor for lowering genetic diversity in wild populations of silvery minnow.

Materials and Methods

Rio Grande silvery minnow eggs were sampled between 5 May 2003 and 21 May 2003. Eggs were captured using an array of modified Moore egg collectors (Altenbach *et al.* 2000) situated at the San Marcial Railroad Bridge Crossing in the middle Rio Grande, New Mexico. A complete list of materials that have been accessioned into the Division of Fishes, Museum of Southwestern Biology is included in this document (Table 9). Eggs have been subsampled for genetic characterization from this collection (Table 9).

DNA isolation, amplification, and characterization follows methods described in **Part I**, with some minor modification (egg membranes will be mechanically ruptured prior to standard DNA isolation). MtDNA-ND4 PCR primers were used successfully in 2000, and these will be used for characterizing 2003 eggs. Hierarchical analyses of molecular variance (AMOVA) (as described in **Part I**) will be used to test for population substructure in mtDNA. In addition, we will continue to develop microsatellite loci for genetic characterization of individual eggs. The success of development of nuclear markers may depend on the stage of development (i.e., an embryo contains much more nuclear DNA than a zygote; mtDNA content is relatively constant throughout development). In a pilot study in 2000, we were able to show that mtDNA offered sufficient polymorphism to detect differences among temporally-spaced eggs samples with high statistical power. In 2003, we will examine two null hypotheses: (i) that egg

samples comprise a random subset of breeding adult (2002) genes; and that (ii) eggs samples do not differ in genetic composition among temporal samples.

Expected Results and Discussion

The 2000 pilot study included four egg samples ($n = 10$ each) spaced over two days. Characterization of mtDNA showed that genetic diversity of these samples differed dramatically ($F_{ST} = 0.19$, $P < 0.001$) between sampling periods, suggesting the possibility that eggs are transported in groups that are more closely related than expected at random. This result is consistent with the interpretation that variance in reproductive success may play an important role for lowering genetic diversity in remnant Rio Grande silvery minnow populations, where groups of genetically related progeny are subject to high mortality as they disperse into a very heterogeneous environment (sensu Hedgecock 1994).

Limited sampling in the year 2000 prevented a critical issue from being addressed, that is, how representative are eggs collected at one site over the entire spawning season of total genetic diversity among breeding adults. We now have unprecedented statistical power to address this question for two reasons: (i) we have extensive egg samples over the entire breeding season (including two spawning spikes), and (ii) we have extensive samples of 2002 and 2003 potential spawners, including wild and hatchery stocks. We expect results of this effort to be critical for guiding egg salvage efforts with respect to strategies that maximize genetic diversity of eggs.

Conservation genetics of Rio Grande silvery minnow, Part V:

Summary of major findings (1999-2003) and implications for management.

Major finding (1): The mean estimate of genetic effective size (N_e) for wild Rio Grande silvery minnow is approximately 100, based on evaluation of genetic change over four generations (1999 – 2002), with over 700 wild individuals assayed. N_e is sufficiently small to warrant concern about extinction from genetic factors. Harmonic mean adult census size (N) over from 1999 to 2001 is approximately 80,470 based on CPUE data (Dudley and Platania, RGSM monitoring project). The ratio $N_e/N = 0.0012$, which indicates that offspring from very few breeding pairs are successfully recruiting in the middle Rio Grande.

Management implications: Minimum N_e required to maintain >95% of genetic variation at a neutrally-evolving genetic locus over the long term is estimated to be $N_e \geq 500$ (Frankham 1995b). To maintain sufficient levels of genetic variation for quantitative traits like body size, fecundity, spawning time, etc, $N_e \geq 5000$ is required (Lande 1995). $N_e/N = 0.0012$ observed over four Rio Grande silvery minnow generations indicate that target goals for adult census size of wild fish (i.e., capable of reproduction) should be between 400,000 and 4,000,000 individuals. This number is obtained by dividing target N_e (e.g., 500 and 5000) by observed $N_e/N = 0.0012$, which yields 416,000 individuals required in the wild to maintain minimal levels of genetic diversity. To preserve quantitative trait variation, approximately 4,166,000 potentially breeding adults need to be maintained in the wild. Propagation and augmentation plans should seek to hold and repatriate as many fishes as required to sustain census numbers within this size range *in*

the wild. Management emphasis should be placed on maintaining environmental conditions that support wild populations of recommended adult census size. For reasons outlined below, *propagation and augmentation should be considered a tool to mitigate catastrophic demographic losses of wild fishes, and not as a substitute for a healthy, wild population of Rio Grande silvery minnow.*

Major finding (2): Captively-reared offspring obtained from small brood-stock revealed lowered allelic diversity at nearly all genetic loci compared to the wild source population. Captively-reared individuals exhibited similar levels of observed heterozygosity and inbreeding coefficients compared to the wild source population.

Management implications: There is a twofold risk to genetic resources associated with captive spawning: (i) loss of genetic diversity at all genetic loci, and (ii) imposition of artificial selection on wild genotypes. Comparison of wild and captive-spawned stocks shows that rare alleles present in wild populations have been lost in captive-spawned stocks. Maximizing the size and geographic sampling of brood stock can reduce these effects, but not eliminate them. Relatively low inbreeding coefficients observed in the captive stock may arise as a consequence of reduced variance in individual reproductive success compared to wild fishes, and suggests the possibility of increasing heterozygosity of captive fishes with a well-designed brood-stock management plan. Our data do not address risks associated with artificial selection in the hatchery and its ecological and genetic effects on wild fish. However, such selection is inevitable and will impact the viability of the wild Rio Grande silvery minnow populations. Thus, captive propagation and augmentation should only be used when no

other management tools are available to prevent the extinction of the Rio Grande silvery minnow.

Major finding (3): Stocks reared from wild-caught eggs maintain allelic diversity but exhibit higher rates of inbreeding than wild stocks.

Management implications: This finding indicates that there are genetic consequences to capturing Rio Grande silvery minnow eggs in the wild, transporting them to rearing facilities, and repatriating these individuals. In Part I of this report, we found that extensive river fragmentation has significant consequences for genetic variation in Rio Grande silvery minnow. Collection, rearing, and repatriation of individuals from wild caught eggs apparently does not ameliorate this effect. Thus, the long-term plan for management of genetic resources should aim to reduce river fragmentation.

Major finding (4): There is no appreciable genetic structure in Rio Grande silvery minnow across sampling localities in three river reaches (Angostura, Isleta, and San Acacia reaches) in the middle Rio Grande, based on analysis of DNA microsatellite and mitochondrial DNA loci.

Management implications: If extinction in the wild is imminent and captive propagation and rearing for repatriation becomes necessary, then brood-stock should be collected throughout the middle Rio Grande, over the current geographic range of the species. There is no need to manage brood stocks for each reach separately. Captive propagation should seek to maximize the number of breeding pairs for production of

captive offspring to maximize the genetic effective population size (N_e), thereby maximizing genetic diversity harbored by captive stocks [see Major Finding (3)].

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Table 1. Sampling locality information, samples sizes, and voucher deposition for Rio Grande silvery minnows collected in the middle Rio Grande, New Mexico, and assayed for genetic variation at microsatellite (μ sats) and mtDNA loci.

Sampling Locality	Generation	River Reach	Latitude	Longitude	Voucher specimens
San Marcial	1999	San Acacia	33° 40' 55"N	106° 59' 33"W	MSB-49213
Former LFCC confluence	2000	San Acacia	33° 35' 19"N	107° 03' 19"W	MSB-49216
San Marcial	2000	San Acacia	33° 40' 55"N	106° 59' 33"W	MSB-49217
Bosque del Apache	2000	San Acacia	33° 47' 46"N	106° 52' 12"W	MSB-49218
San Antonio	2000	San Acacia	33° 55' 10"N	106° 51' 04"W	MSB-49219
San Acacia	2001	San Acacia	34° 14' 36"N	106° 53' 58"W	MSB-49221
Los Lunas	2001	Isleta	34° 39' 54"N	106° 44' 59"W	*
Angostura	2002	Angostura	35° 22' 49"N	106° 29' 57"W	*

*No vouchers available, fin clips only

Table 2. Summary statistics for microsatellite and mtDNA data, tabulated by collection locality and generation. Sample size (n), Hardy-Weinberg expected heterozygosities (H_E), observed (direct count) heterozygosities, and the mean number of alleles per locus are reported for microsatellites. For mtDNA, h is Nei's (1987) nucleon diversity, and the total number of haplotypes identified in a sample is reported. Sampling localities correspond to Table 1, and Figure 1.

Sampling Locality	Generation	Microsatellites				MtDNA		
		n	H_E	H_O	Mean alleles/locus	n	h	haplotypes
San Marcial	1999	46	0.679	0.607	8.7	42	0.374	5
Former LFCC confluence	2000	29	0.752	0.658	10.9	33	0.119	3
San Marcial	2000	60	0.734	0.689	13.0	45	0.351	4
Bosque del Apache	2000	58	0.684	0.527	10.7	8	0.536	2
San Antonio	2000	47	0.743	0.659	11.6	29	0.589	5
San Acacia	2001	64	0.699	0.623	12.1	26	0.412	6
Los Lunas	2001	64	0.689	0.542	11.7	42	0.489	9
Angostura	2002	29	0.744	0.720	9.4	24	0.667	5
Averaged across populations		49.6	0.716	0.628	11.02	31.1	0.442	4.88

Table 3. Temporal changes in adult census and genetic effective sizes over three generations of Rio Grande silvery minnow in the wild. Pair-wise contrasts across generations were used to estimate harmonic mean adult census size (N), variance genetic effective size (N_e) calculated from temporal allele frequency changes at seven microsatellite loci (95% CIs in parentheses), and variance effective size calculated from temporal changes in haplotype frequencies at the mtDNA – ND4 locus (and 95% CIs). Infinity symbol (∞) indicates values of N_e that is indistinguishable from a population of infinite size (Waples 1989). The ratio N_e/N is expected to be unity in a Wright-Fisher idealized population.

Contrast	Harmonic mean N	N_e (μ sats)	N_e (mtDNA)	N_e/N (μ sats)	N_e/N (mtDNA)
1999 vs. 2000	401409	41 (12, 91)	92 (7, ∞)	0.0001	0.0002
1999 vs. 2001	59698	93 (45, 299)	∞	0.0016	---
2000 vs. 2001	56667	73 (44, 181)	552 (24, ∞)	0.0013	0.0097

Table 4. Summary statistics for microsatellite and mtDNA – ND4 loci screened from wild Rio Grande silvery minnow captured in 2002. Sampling localities are given: Angostura, Isleta and San Acacia and over all populations. Sample size (n), Expected heterozygosity (H_E), observed heterozygosity (H_O), mean number of alleles per locus and average weighted inbreeding co-efficient (F_{IS}) are give overall loci. For ND4 the observed number of haplotypes and the gene diversity (h) are given.

Sampling Locality	Microsatellites					MtDNA- ND4		
	n	H_E	H_O	Mean alleles/locus	F_{IS}	n	h	haplotypes
Angostura	67	0.774	0.610	14.90	0.213	68	0.681	6
Isleta	121	0.752	0.580	17.10	0.229	109	0.670	8
San Acacia	201	0.716	0.565	21.50	0.211	200	0.613	8
All Populations	389	0.744	0.578	23.90	0.233	377	0.641	8

Table 5. Mitochondrial ND4 haplotype frequencies among wild Rio Grande silvery minnow sampled in 2002. Frequencies are give for each reach of the Rio Grande (Angostura, Isleta and San Acacia) and across all populations.

Haplotype	Reach			All Pops 2002
	Angostura	Isleta	San Acacia	
A	0.500	0.501	0.575	0.541
C	0.191	0.220	0.200	0.204
D	0.191	0.165	0.125	0.357
E	-	0.009	0.015	0.012
F	0.059	0.073	0.050	0.058
J	-	-	0.005	0.003
K	0.004	0.029	0.030	0.032
O	0.015	-	-	0.003

Table 6. Temporal changes in adult census and genetic effective sizes compared across 1999, 2000, 2001 and 2002 generations of Rio Grande silvery minnow in the wild. Pair-wise contrasts across generations were used to estimate variance genetic effective size (N_e) calculated from temporal allele frequency changes at seven microsatellite loci (95% CIs in parentheses). Microsatellites *Ca1*, *Ca6*, and *Ca8* were not used in these comparisons because they were not screened in wild fish prior to 2002.

Contrast	N_e μ sats (95% CIs)
1999 vs. 2002	139 (75, 228)
2000 vs. 2002	121 (81, 158)
2001 vs. 2002	55 (36, 74)
Average N_e	105

Table 7. Summary statistics for microsatellite and mtDNA – ND4 loci screened from wild Rio Grande silvery minnows sampled in 2002 and hatchery-spawned and reared (ABQ BioPark) (Hatch 2000), and hatchery-reared fish from wild-caught eggs (Hatch 2002, Hatch 2003). Sample size (n), expected heterozygosity (H_E), observed heterozygosity (H_O), mean number of alleles per locus, mean allelic richness (based on the minimum sample size of 58) and average weighted inbreeding co-efficient (F_{IS}) are give over all loci. For ND4 the observed number of haplotypes and the gene diversity (h) are given.

Sampling Locality	Microsatellites						MtDNA- ND4		
	n	H_E	H_O	Mean alleles/locus	Mean Allelic Richness	F_{IS}	n	h	haplotypes
Hatch 2000	64	0.711	0.570	11.80	11.60	0.199	58	0.460	5
Hatch 2002	178	0.778	0.502	20.60	15.95	0.356	157	0.624	8
Hatch 2003	81	0.751	0.453	15.60	14.43	0.398	81	0.703	8
Wild 2002	389	0.744	0.578	23.90	15.47	0.223	377	0.641	8

Table 8. Mitochondrial ND4 haplotype frequencies among wild Rio Grande silvery minnows sampled in 2002: hatchery-spawned and reared (ABQ BioPark) (Hatch 2000), and hatchery-raised fish from wild-caught eggs (Hatch 2002, Hatch 2003).

Haplotype	Hatchery			Wild
	2000	2002	2003	
A	0.724	0.573	0.481	0.541
C	0.052	0.197	0.222	0.204
D	-	0.051	0.049	0.357
E	0.034	0.064	0.012	0.012
F	0.069	0.064	0.136	0.058
J	-	-	-	0.003
K	0.121	0.032	0.049	0.032
M	-	0.013	0.037	-
N	-	0.006	-	-
O	-	-	-	0.003
P	-	-	0.012	-

Table 9. Cyprinid early life stages collected during 2003 Rio Grande silvery minnow Spawning Periodicity Study (directed by Steven P. Platania; Museum of Southwestern Biology Accession ACC2003-V:7). Two hundred thirty three eggs have been subsampled for genetic analysis.

MSB Field No.	Sample Description	N	n subsampled	Collect date	Time start	Time Stop
EGG03-002	Cyprinidae eggs	5		5/5/2003	1440	1640
EGG03-003	Cyprinidae eggs	816	30	5/6/2003	1015	1345
EGG03-004	Cyprinidae eggs	78		5/7/2003	1030	1430
EGG03-016	Cyprinidae eggs	6		5/13/2003	1430	1630
EGG03-018	Cyprinidae eggs	1		5/14/2003	1000	1200
EGG03-039	Cyprinidae eggs	77	30	5/20/2003	2400	100
EGG03-040	Cyprinidae eggs	58		5/20/2003	330	400
EGG03-041	Cyprinidae eggs	568		5/20/2003	605	702
EGG03-042	Cyprinidae eggs	557		5/20/2003	815	900
EGG03-043	Cyprinidae eggs	1235	30	5/20/2003	900	1000
EGG03-044	Cyprinidae eggs	1018		5/20/2003	1000	1100
EGG03-045	Cyprinidae eggs	1354		5/20/2003	1100	1200
EGG03-046	Cyprinidae eggs	1221		5/20/2003	1200	1300
EGG03-046	Larva/juv.	1		5/20/2003	1200	1300
EGG03-047	Cyprinidae eggs	742	30	5/20/2003	1300	1400
EGG03-047	Larva/juv.	1		5/20/2003	1300	1400
EGG03-048	Cyprinidae eggs	1014		5/20/2003	1400	1500
EGG03-048	Larva/juv.	1		5/20/2003	1400	1500
EGG03-049	Cyprinidae eggs	1021		5/20/2003	1500	1600
EGG03-050	Cyprinidae eggs	450		5/20/2003	1600	1700
EGG03-051	Cyprinidae eggs	710	30	5/20/2003	1700	1800
EGG03-052	Cyprinidae eggs	369		5/20/2003	2045	2200
EGG03-052	Larva/juv.	1		5/20/2003	2045	2200
EGG03-053	Cyprinidae eggs	434		5/20/2003	2200	2300
EGG03-054	Cyprinidae eggs	293	30	5/20/2000	2300	2400
EGG03-055	Cyprinidae eggs	208	30	5/21/2003	2400	100
EGG03-056	Cyprinidae eggs	183		5/21/2003	100	200
EGG03-057	Cyprinidae eggs	125		5/21/2003	730	800
EGG03-058	Cyprinidae eggs	74		5/21/2003	800	830
EGG03-059	Cyprinidae eggs	150	23	5/21/2003	830	930

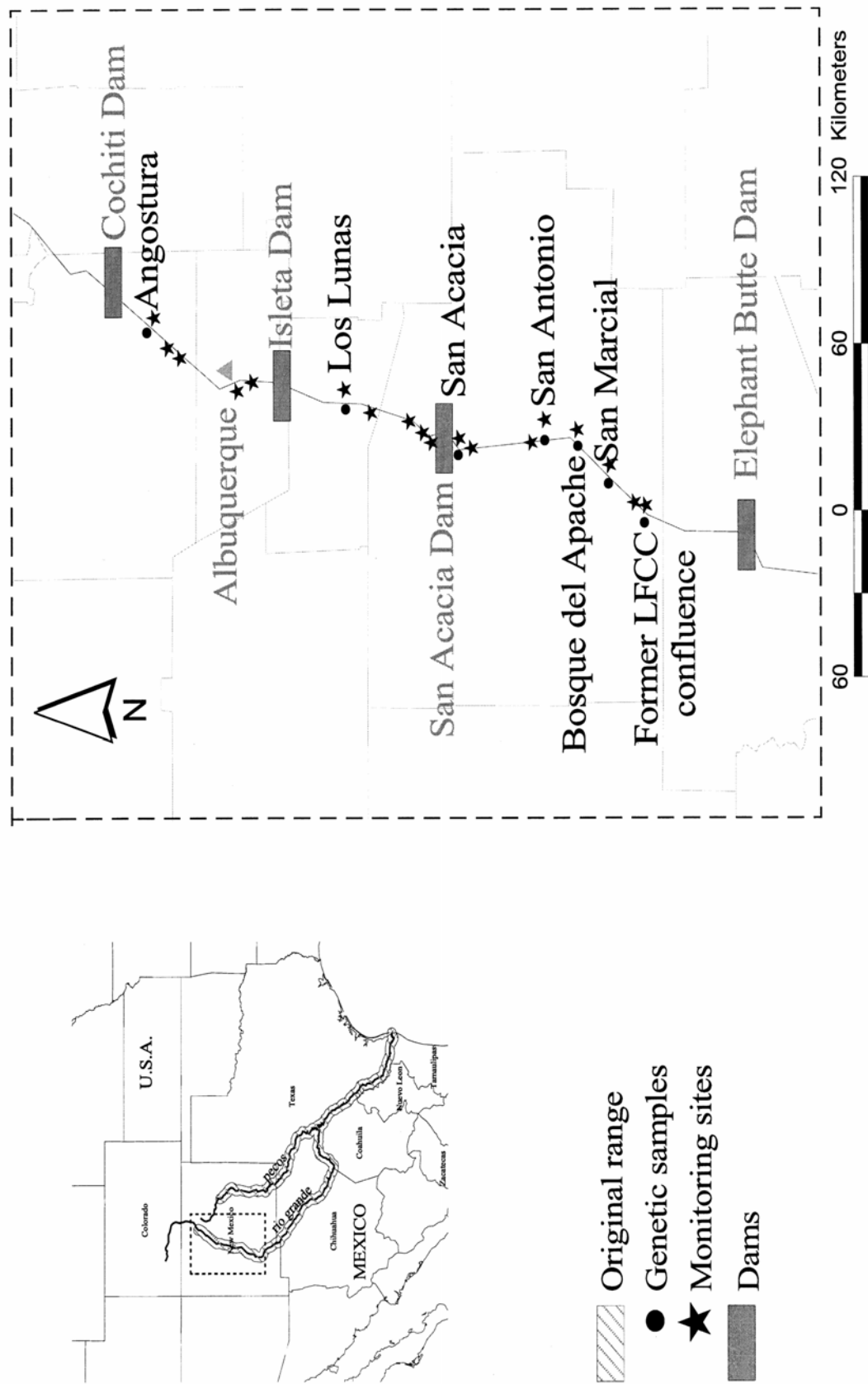


Figure 1. Map of ecological monitoring sites and genetic sampling localities in the middle Rio Grande, New Mexico, USA. The inset map depicts the study site in dashed lines, and the historical geographic range of Rio Grande silvery minnow in the Rio Grande and Pecos River drainages.

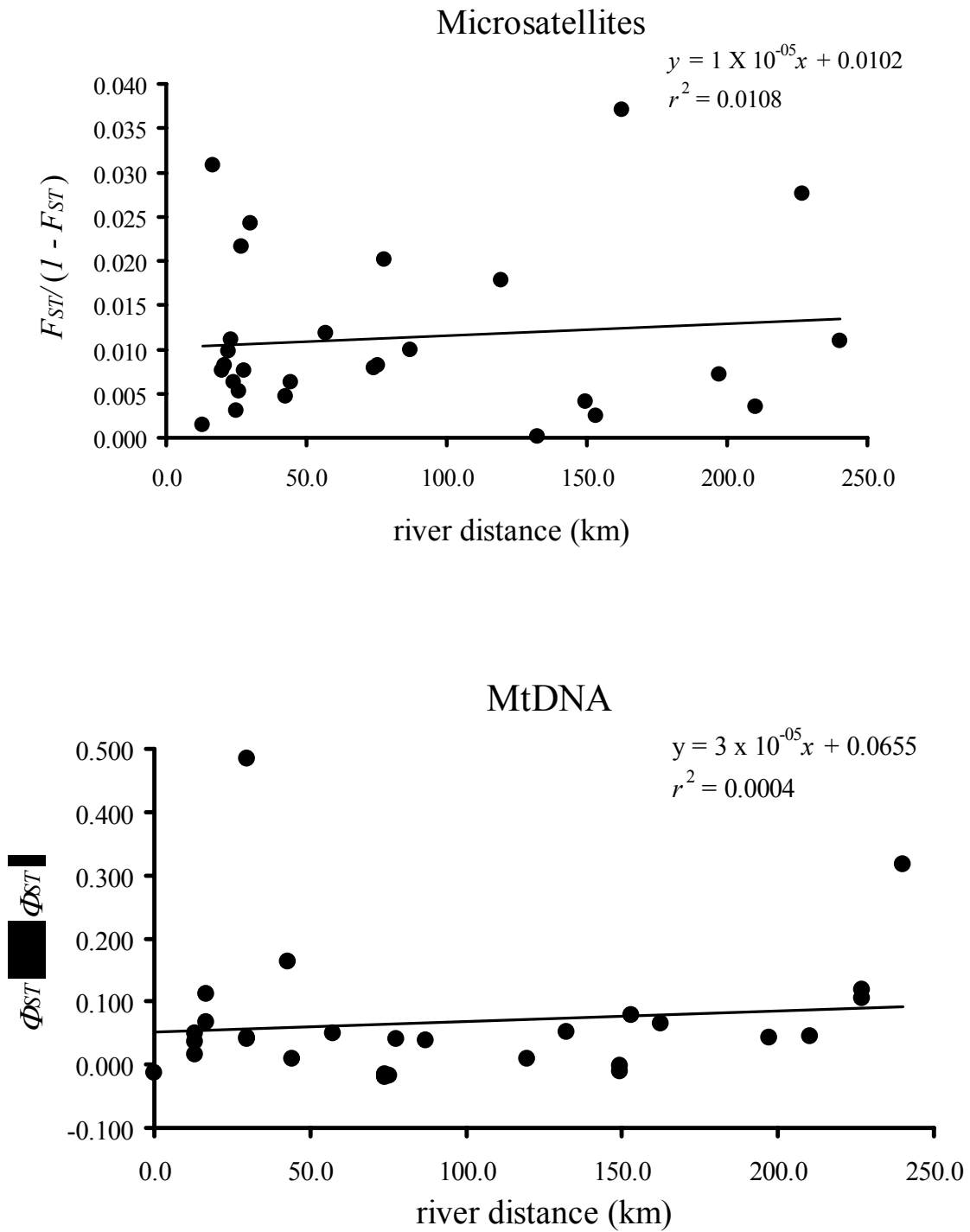


Figure 2. Bivariate plots of genetic distance on geographic distance for microsatellite and mtDNA data sets, respectively. Best-fit equations generated from ordinary least squares regression and variance explained (r^2) are in the upper right-hand corner. Neither slope was significantly different from zero using an F -test with 1, 7 degrees of freedom.

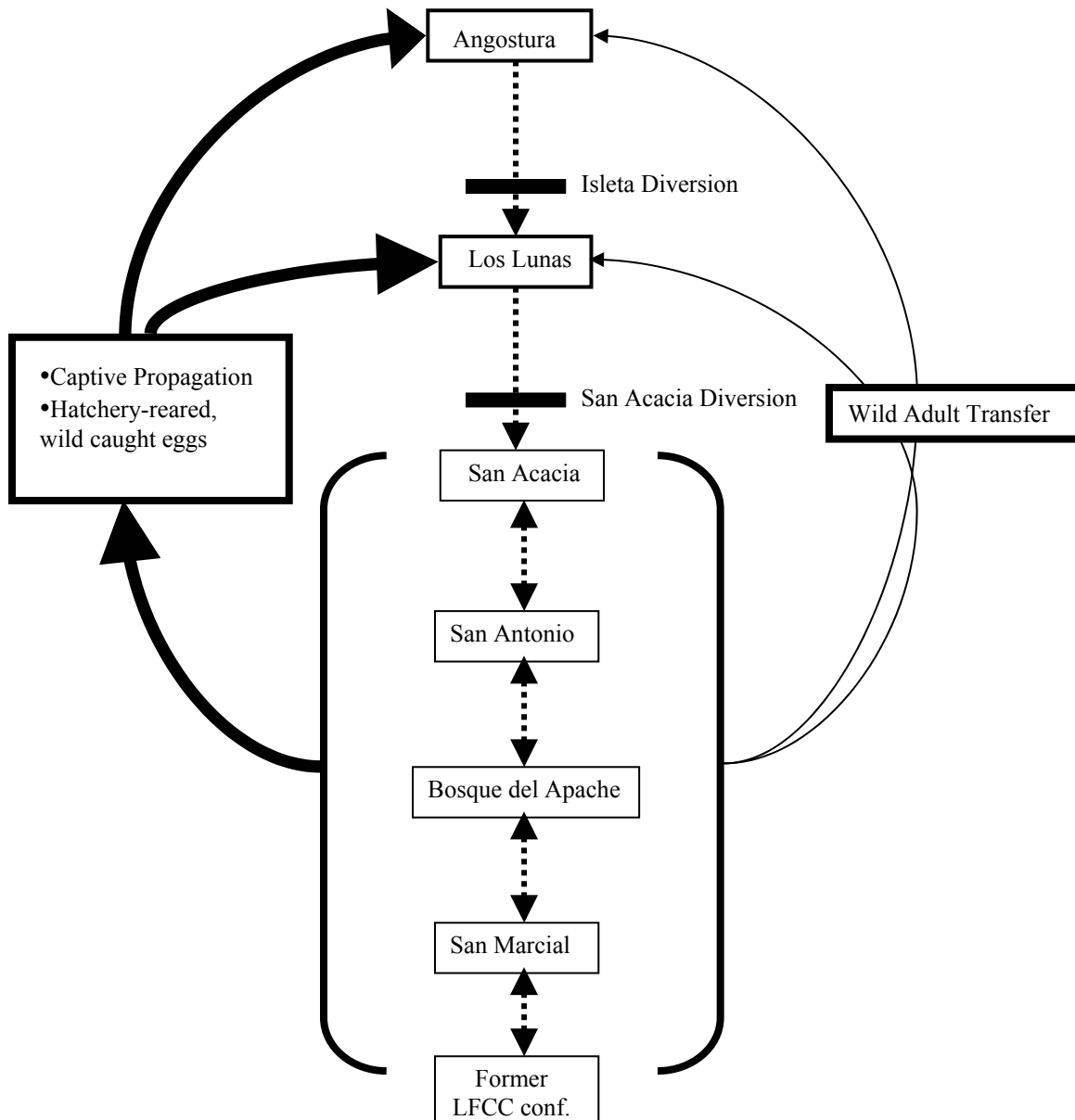


Figure 3. A conceptual model of dispersal and gene flow in the highly fragmented middle Rio Grande. Sampling localities are depicted from north (at the top of the page) to south. Dashed lines indicate natural movement of individuals (eggs, larvae, and/or adults) between sampling localities. Note that movement among river reaches is downstream only. Solid lines indicate artificial movement of wild and hatchery propagated Rio Grande silvery minnow as a management strategy. Relative numbers of individuals moved are indicated by the thickness of solid lines. For example, the number of individuals repatriated from hatcheries is much greater than the number of wild fish transported to northern reaches. The source population for all transfers is in the San Acacia reach, which currently supports the largest natural populations of Rio Grande silvery minnow.

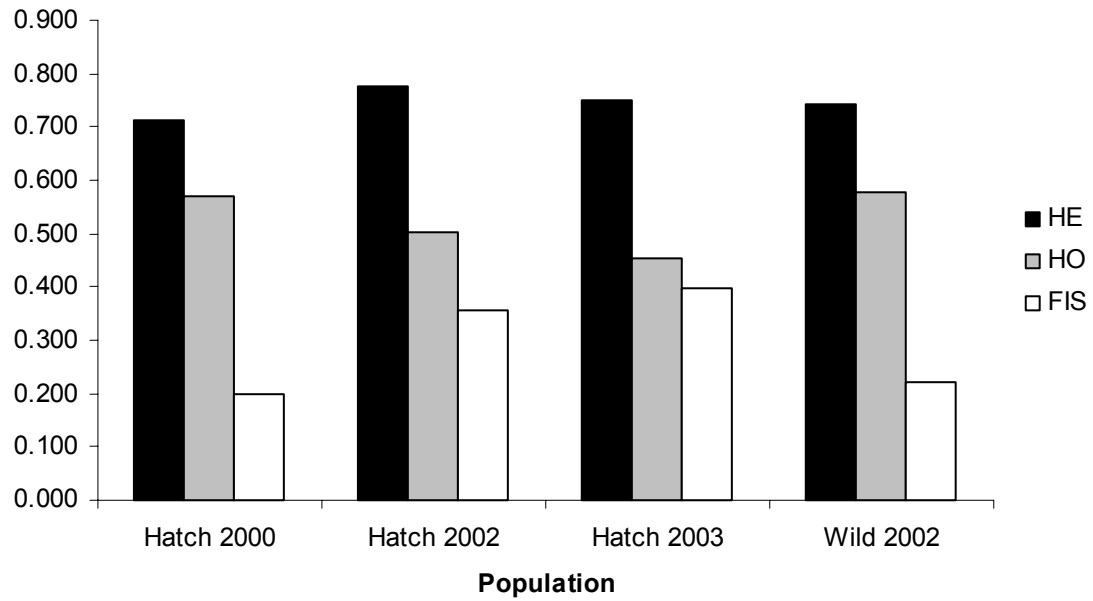


Figure 4. Summary statistics across 10 microsatellite loci for Rio Grande silvery minnow wild 2002 populations, captively-spawned and reared stock (Hatch 2000) and from captively-propagated wild caught eggs (Hatch 2002, 2003). Expected heterozygosity (H_E), observed heterozygosity (H_O) and average weighted inbreeding coefficient (F_{IS}) are given.

Appendix I: Manuscript in press in the peer-reviewed journal *Conservation Genetics* describing microsatellite loci developed for Rio Grande silvery minnow.

**Variable microsatellite markers amplify across divergent lineages of cyprinid fishes
(subfamily Leuciscinae)**

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Cyprinidae is arguably the most species-rich, primary fish family in the world with over 2000 species distributed on four continents (Helfman *et al.* 1997). The family includes economically and scientifically important freshwater species such as carps, minnows, barbs, and zebrafishes. Thirty-nine of the 270 or so cyprinid species that occur in North America are threatened or endangered, and 29 of those occupy arid river basins in the southwestern US (US Fish and Wildlife Service 2002). Conservation efforts for threatened and endangered cyprinids are increasingly dependent on genetic data for guidance in defining conservation units and designing captive propagation plans for endangered taxa (Hedrick *et al.* 2000). In this study, we present primer sequences and

PCR conditions for eight polymorphic DNA microsatellite loci developed for cyprinids. We tested each primer pair for ability to amplify representatives of three genetically divergent North American cyprinid lineages (all in the subfamily Leuciscinae - Cavender and Coburn 1992), and summarize previously published cross-amplification results and microsatellite allele frequency data from one European cyprinid fish lineage (Salguiero *et al.* 2003). Two of the cyprinid fish species examined in this study are in danger of extinction.

Microsatellite loci were isolated from a partial genomic library constructed for the common shiner, *Luxilus cornutus*, which is an abundant cyprinid found in the northeastern US. Nucleic acids were obtained through density gradient ultracentrifugation purification (Dowling *et al.* 1996) and then digested with the restriction endonuclease *DpnII* (New England Biolabs). Resulting fragments were size-selected in the range of approximately 200 - 800 base pairs (bp) by electrophoresis through a low-melting point agarose gel and purified using Prep-A-Gene (Bio-Rad) DNA purification kits. Size-selected fragments were ligated into pUC18, and heat-shock transformed into *E. coli* strain DH5 α following Sambrook *et al.* (1989). The resulting partial genomic library consisted of 1920 clones that were transferred to nylon membranes. Membranes were screened for three classes of synthetic oligonucleotide probes: di- ([CA]₁₅, [GA]₁₅); tri- ([CCT]₇, [ATT]₇); and tetranucleotide ([GATA]₅, [GAGC]₅, [GTCA]₅, [CTCA]₅, [GACT]₅, [CTAG]₅, [GCAT]₅, [GCAC]₅) repeats (Sigma-GenoSys). The tri- and tetranucleotide screening identified ten positive clones; subsequent nucleotide sequencing of nine clones revealed a diversity of tri- and

tetranucleotide repeats. Dinucleotide screening yielded a minimum of 38 positive clones. Nucleotide sequencing of 14 positive colonies indicated dinucleotide repeats.

Polymerase chain reaction (PCR) primers were designed from unique nucleotide sequence regions flanking microsatellites using the computer program OLIGOTM (Macintosh vers. 4.0, National Biosciences). Annealing temperatures and MgCl₂ conditions were optimized for eight primer pairs (Table 1), using genomic DNAs isolated from one *L. cornutus* and one *L. chrysocephalus* individual as templates for PCR. A locus was considered optimized when PCR produced one or two strongly amplified bands with minimal stutter bands and other ancillary products. PCR amplification was carried out in 10 µl volumes containing 1 µl (50- 200 ng) sample DNA, 1µl 10X reaction buffer (500 mM KCl, 100 mM Tris [pH 9.0], 10% Triton-X 100), 200 µM of each dNTP, 1 mM – 2 mM MgCl₂, 5 pmols of each PCR primer, and 0.1 µl *Taq* DNA polymerase (isolated from a clone, Pluthero 1993). Initially, PCR products were labeled by incorporating [α^{32} P]-dATP. In subsequent PCR experiments with other cyprinid taxa, primers were end-labelled with [γ^{32} P]-dATP. PCR amplification consisted of 25 - 30 cycles of denaturation at 94°C for 30 sec, annealing at 56 - 62°C for 30 sec, and extension at 72°C for 30 sec, preceded by an initial denaturation step at 94°C for 2 min. PCR products were analysed on 6% polyacrylamide gels (Sequagel, National Diagnostics) and visualized by autoradiography.

Eight pairs of microsatellite primers (Table 1) developed from the *L. cornutus* library were tested for their ability to amplify microsatellites in cyprinid fishes. Three species from North American were screened; *Platygobio gracilis* (flathead chub),

Rhinichthys cataractae (longnose dace), and the federally endangered *Hybognathus amarus* (Rio Grande silvery minnow). DNA isolation, PCR, and scoring were conducted as above. However, annealing temperature was held constant at 48 C and MgCl₂ held constant at 2.0 mM per reaction across all species. Genotype data were used to compute allele frequencies and observed and Hardy-Weinberg expected heterozygosities. For comparison, we included data on amplification and variability of these eight primers in the endangered *Anaecypris hispanica* on the Iberian Peninsula (Salguiero *et al.* 2003).

Despite some variability in expected product lengths and heterozygosity, microsatellite primer pairs generated from *Luxilis* produced well-resolved, polymorphic, and scorable products for five of eight loci, on average, for the other cyprinids examined (Table 2). Identical PCR conditions were employed but size ranges of alleles often did not overlap across loci (Table 2), which indicates that subsets of these microsatellite loci are potentially good candidates for multiplex PCR. Allelic diversity (i.e., the number of alleles, 0 = no amplification) was tested for association among taxa by Pearson correlation analysis. Significant correlation ($r = 0.84$; $p < 0.05$) of allelic diversity was observed for *Rhinichthys* and *Platygobio*, which may reflect their relatively recent common ancestry (Simons and Mayden 1998). Patterns of allelic diversity in *Hybognathus* and *Anaecypris* were not significantly correlated with other study species. Thus, allelic variability in one species is probably not a good predictor of variability in other cyprinid taxa. However, microsatellite loci developed in this work appear to be sufficiently variable and reliable for population and conservation genetic applications for a diverse group of leuciscin and phoxinin cyprinid fishes.

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Table 1. Microsatellite DNA loci obtained from the common shiner *Luxilus cornutus*. Product lengths in base pairs (bp) were determined from clones obtained from a partial genomic library. Microsatellite loci were originally developed for hybridization study of *L. cornutus* and *L. chrysocephalus*, but preliminary genetic screening indicated shared alleles even among geographically distant samples. Allele range sizes are not available. Repeat sequence indicates the repeat motif [in brackets] and the number of uninterrupted copies observed in the cloned allele. DNA sequences of clones are listed in GenBank under sequential accession numbers AY318777 – AY318784.

Locus	Primer Sequences (5' – 3')	Product size (bp)	Repeat sequence	Annealing Temp. (°C)	[MgCl ₂]
<i>Lco1</i>	CAC GGG ACA ATT TGG ATG TTT TAT AGG GGG CAG CAT ACA AGA GAC AAC	159	[GATA] ₈ GGC TA[GATA] ₂	60	1.5
<i>Lco2</i>	ATT TTT AGG AGT GAT GTT CAG CAT CAA GTG TGT CAT TGA GGA AGT GAG	190	[TGTC] ₆ [TATC] ₃ TATA [TG(A)TC] ₁₆	53	2.25
<i>Lco3</i>	GCA GGA GCG AAA CCA TAA AT AAA CAG GCA GGA CAC AAA GG	225	[TG] ₉	60	2.5
<i>Lco4</i>	ATC AGG TCA GGG GTG TCA CG TGT TTA TTT GGG GTC TGT GT	197	[GT] ₅ ATTTT [GT] ₅ [GA] ₁₁	60	2.25
<i>Lco5</i>	TTA CAC AGC CAA GAC TAT GT CAA GTG ATT TTG CTT ACT GC	118	[CAGA] ₂ [CA] ₁₄	57	2.5
<i>Lco6</i>	CCA CAC AGA GCA TTT GTA TT GTG GCT GAT TTC ATT ATT CA	130	[CA] ₁₂	57	2.5
<i>Lco7</i>	AGA AGG TAA GAG GAA TGT GT TGA GGT AGA TAG CCA AGT TT	129	[GT] ₂ GTTT[GT] ₈ GA[GT] ₃	57	2.5
<i>Lco8</i>	GCT TTG AAC ACT TGG CTT AT AGG CTG GAC TGA ATC ACT TC	255	[TC] ₂₅ [TG] ₅	63	2.25

Table 2. Number of alleles, size range of alleles in base pairs (bp), observed (direct count) heterozygosity (H_O), and Hardy-Weinberg expected heterozygosities (H_E), scored in cross-species amplification experiments using eight loci developed from *Luxilus cornutus*. Number of individuals examined (n) is indicated for each species. Allele frequency data and PCR conditions for *Anaocypris hispanica* are described in Salguiero *et al.* (2003).

Locus	<i>Hybognathus amarus</i> (n = 12)				<i>Rhinichthys cataractae</i> (n = 12)				<i>Platygobio gracilis</i> (n = 12)				<i>Anaocypris hispanica</i> (n = 310)			
	alleles	size range (bp)	H_O	H_E	alleles	size range (bp)	H_O	H_E	alleles	size range (bp)	H_O	H_E	alleles	size range (bp)	H_O	H_E
<i>Lco1</i>	10	200 – 348	1.00	0.94	12	258 – 306	1.00	0.94	12	236 – 270	1.00	0.93	38	300 – 444	0.79	0.94
<i>Lco2</i>	---	---	---	---	1	254	0.00	0.00	---	---	---	---	---	---	---	---
<i>Lco3</i>	6	235 – 265	1.00	0.84	3	242 – 244	0.58	0.55	---	---	---	---	22	241 – 303	0.72	0.76
<i>Lco4</i>	2	228 – 237	0.36	0.42	3	233 – 239	0.42	0.04	3	230 – 240	0.58	0.54	33	250 – 320	0.80	0.90
<i>Lco5</i>	2	130 – 153	0.08	0.08	11	146 – 178	1.00	0.89	14	150 – 180	1.00	0.93	---	---	---	---
<i>Lco6</i>	5	160 – 189	0.58	0.59	---	---	---	---	5	180 – 182	0.75	0.79	1	N/A	0.00	0.00
<i>Lco7</i>	8	137 – 163	1.00	0.80	3	220 – 224	0.10	0.10	3	154 – 160	0.33	0.49	---	---	---	---
<i>Lco8</i>	5	278 – 302	0.83	0.80	4	288 – 294	0.75	0.73	---	---	---	---	---	---	---	---

--- scorable products were not obtained; N/A – data not available

Appendix II: Summary statistics for ten microsatellite and mtDNA – ND4 loci screened from wild Rio Grande silvery minnow collected in 2002 (Angostura, Isleta, San Acacia), and hatchery-spawned and reared (ABQ BioPark) (Hatch 2000), and hatchery-reared fish from wild-caught eggs (Hatch 2002, Hatch 2003). Expected heterozygosity (H_E), observed heterozygosity (H_O), number of alleles (total number of alleles across all populations is given in parenthesis), allele size range, allelic richness and average weighted inbreeding co-efficient (F_{IS}) (significant F_{IS} values are given in bold) are give for all loci. For ND4 the observed number of haplotypes and the gene diversity (h) are given.

Locus	Population	Hatch 2000	Hatch 2002	Hatch 2003	Angostura	Isleta	San Acacia	Combined WILD
	Sample size	64	178	81	67	121	201	389
Lco1	H_E	0.948	0.962	0.965	0.959	0.964	0.965	0.965
	H_O	0.821	0.576	0.722	0.862	0.775	0.838	0.822
	No Alleles (61)	27	41	36	35	42	50	55
	Size Range	241-344	201-344	209-348	221-342	201-348	205-348	201-348
	Allelic Richness	26.745	32.733	32.542	33.133	34.424	35.335	35.824
	F_{IS}	0.135	0.401	0.254	0.102	0.197	0.132	0.148
Lco3	H_E	0.752	0.789	0.819	0.796	0.776	0.764	0.777
	H_O	0.807	0.566	0.541	0.810	0.819	0.774	0.794
	No Alleles (16)	8	14	12	11	12	13	14
	Size Range	241-257	235-265	237-261	235-257	235-259	237-263	235-263
	Allelic Richness	7.895	10.600	11.490	10.690	10.392	10.362	10.646
	F_{IS}	-0.073	0.283	0.341	-0.017	-0.056	-0.014	-0.022
Lco4	H_E	0.561	0.567	0.626	0.683	0.670	0.663	0.684
	H_O	0.241	0.442	0.310	0.582	0.647	0.568	0.595
	No Alleles (13)	5	11	11	8	9	10	12
	Size Range	231-237	226-237	221-234	227-234	226-237	221-237	221-237
	Allelic Richness	5.000	8.511	9.861	7.224	7.946	7.859	8.513
	F_{IS}	0.573	0.222	0.507	0.149	0.034	0.144	0.13
Lco5	H_E	0.418	0.558	0.520	0.699	0.626	0.509	0.593
	H_O	0.458	0.672	0.529	0.587	0.436	0.406	0.446
	No Alleles (8)	4	5	4	5	6	8	8
	Size Range	130-133	129-133	130-133	130-134	129-134	129-136	129-136
	Allelic Richness	3.915	4.280	3.999	4.981	5.449	6.770	5.642
	F_{IS}	-0.095	-0.205	-0.017	0.160	0.305	0.203	0.249

Locus	Population	Hatch 2000	Hatch 2002	Hatch 2003	Angostura	Isleta	San Acacia	All Wild
Lco6	H_E	0.651	0.818	0.781	0.745	0.650	0.626	0.655
	H_O	0.696	0.475	0.456	0.524	0.504	0.434	0.472
	No Alleles (26)	12	24	18	18	17	20	23
	Size Range	168-189	163-187	166-189	164-189	162-189	166-189	162-189
	Allelic Richness	11.924	18.570	16.225	17.082	13.956	15.525	17.356
	F_{IS}	-0.071	0.420	0.418	0.298	0.225	0.307	0.28
Lco7	H_E	0.818	0.884	0.883	0.809	0.824	0.797	0.811
	H_O	0.625	0.512	0.329	0.469	0.537	0.548	0.531
	No Alleles (24)	10	22	18	13	14	18	19
	Size Range	137-163	137-169	137-164	141-169	137-169	137-169	137-169
	Allelic Richness	9.962	16.209	17.000	12.325	12.084	13.385	16.667
	F_{IS}	0.238	0.422	0.629	0.423	0.349	0.313	0.345
Lco8	H_E	0.788	0.882	0.857	0.846	0.884	0.877	0.877
	H_O	0.625	0.425	0.444	0.613	0.575	0.633	0.612
	No Alleles (27)	14	20	12	14	17	20	21
	Size Range	274-312	254-310	272-312	274-310	274-312	270-310	270-312
	Allelic Richness	13.325	15.905	10.663	13.578	14.412	14.364	14.967
	F_{IS}	0.208	0.519	0.483	0.277	0.350	0.278	0.303
CA1	H_E	0.547	0.531	0.366	0.460	0.366	0.252	0.329
	H_O	0.156	0.165	0.050	0.164	0.033	0.131	0.106
	No Alleles (18)	4	13	6	8	8	7	11
	Size Range	75-91	74-96	75-92	75-95	75-95	75-95	75-95
	Allelic Richness	3.844	8.671	5.142	7.03	5.918	4.977	7.184
	F_{IS}	0.716	0.690	0.864	0.644	0.910	0.482	0.678
CA6	H_E	0.685	0.828	0.750	0.805	0.811	0.741	0.786
	H_O	0.781	0.638	0.638	0.821	0.736	0.658	0.711
	No Alleles (22)	10	18	11	11	14	16	19
	Size Range	201-221	191-225	195-217	185-221	191-223	189-221	185-223
	Allelic Richness	9.614	14.155	10.526	10.418	11.406	10.964	12.579
	F_{IS}	-0.142	0.230	0.150	-0.020	0.093	0.111	0.096

Locus	Population	Hatch 2000	Hatch 2002	Hatch 2003	Angostura	Isleta	San Acacia	Combined WILD
CA8	H_E	0.938	0.959	0.944	0.938	0.948	0.967	0.958
	H_O	0.491	0.542	0.516	0.667	0.739	0.663	0.689
	No Alleles (60)	24	38	28	26	32	53	57
	Size Range	101-217	101-222	101-218	104-222	104-222	104-229	104-229
	Allelic Richness	23.778	29.870	26.798	26	26.496	38.505	32.957
	F_{IS}	0.478	0.435	0.455	0.291	0.220	0.315	0.281
F_{IS} All		0.199	0.356	0.398	0.213	0.229	0.211	0.223
ND4	No Alleles (11)	5 (58)	8 (157)	8 (81)	6 (68)	7 (109)	7 (200)	8 (377)
	Gene Diversity (h)	0.460	0.624	0.703	0.681	0.670	0.613	0.641